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Original Article

Expression and purification of SARS-CoV-2 receptor binding domain in *Escherichia coli* for diagnostic and therapeutic purposes

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Abstract

Background and purpose: SARS-CoV-2 causes a severe respiratory disease known as COVID-19 and is responsible for a global viral pandemic. The SARS-CoV-2 receptor binding domain (RBD) is located on the spike protein, which identifies and binds to the angiotensin-converting enzyme 2 (ACE2) receptor. The RBD is an important target for developing virus-neutralizing antibodies, vaccines, and inhibitors.
Experimental approach: In this study, recombinant SARS-CoV-2 RBD was expressed in *E. coli BL21 (DE3)* and purified and its binding activity was determined. Purification was conducted using the Ni-NTA column. ELISA. flow cytometry assays were set to evaluate the binding ability of recombinant RBD to different anti-RBD antibodies and native ACE2 receptors on HEK293A cells, respectively.
Findings/Results: The SDS-PAGE analysis revealed the corresponding band at 27 kDa in the culture after induction with 0.7 mM IPTG, while the corresponding band was not observed in the culture without IPTG induction. ELISA results showed that antibodies produced in the human sera could bind to the recombinant RBD protein and the commercial anti-RBD antibody. Also, flow cytometry analysis revealed that the recombinant RBD could bind to human ACE2 on the surface of HEK293A cells.
Conclusion and implication: Our outcomes displayed that the recombinant RBD expressed in the *E. coli* strain has biological activity and can be used as an antigen for the development of diagnosis kits and vaccines as well as a tool for screening drugs against SASR-CoV-2.

Keywords: Angiotensin-converting enzyme 2; Gene cloning; Purification; Receptor binding domain; Severe-acute respiratory syndrome coronavirus 2.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by a new coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and now is widespread around the globe readily, causing a worldwide health crisis (1). More than 129 million people have been infected and 14.9 million excess deaths associated with the COVID-19 pandemic in 2020 and 2021 worldwide (2). COVID-19 disturbs the respiratory tract, presenting with symptoms ranging from common colds to pneumonia, including common cold symptoms, diarrhea, nasal congestion, dry coughs, fevers, fatigue, and sore throats (3,4). There is a crucial need to design efficacious preventive vaccines and therapeutics versus SARS-CoV-2.

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The SARS-CoV-2 particle, like other coronaviruses, is spherical and it has four structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N) (5). There are two subunits namely S1 and S2, in the S protein, which is a transmembrane glycosylated protein with 1273 amino acids (6). The S1 subunit comprises a receptor binding domain (RBD) that identifies and interacts with the host receptor angiotensin-converting enzyme 2 (ACE2), whereas the S2 subunit intercedes viral cell membrane fusion (7). The role RBD plays in the entry of viruses into host cells and the production of neutralizing antibodies triggered by infection makes it an attractive target for vaccine development (8).

RBD comprises 220 amino acid residues with two N-glycosylation sites and nine cysteine residues (9). A 34 kDa apparent molecular mass has been determined for RBD, while a 27 kDa amino acid sequence has been determined for it (10). Analysis of RBD reveals both N- and O-glycosylation patterns that play a vital role in immunogenicity, stability, and protein folding (11). RBD has five strands which form a central twisted antiparallel *β*-sheet decorated with loops and secondary structure elements (12). Since the outbreak of SARS-CoV in 2003, eukaryotic systems have been used to produce recombinant SARS-CoV RBD including yeast (13), Chinese hamster ovary (14), HEK293 (15), and baculovirus (16). The current expression systems exhibit significant limitations in the development of vaccines, diagnostic, kits, or therapeutics for therapeutic and diagnostic purposes due to their high cost and low yield (17). Furthermore, prokaryotic systems' potential for recombinant RBD production remains underutilized and underexploited. The utilization of bacterial systems for heterologous protein expression offers significant advantages including rapid production timelines, cost-effectiveness, and scalability (18). Principally, *Escherichia coli* (*E. coli*) is one of the most prevalent bacterial hosts for heterologous protein expression (18). Certain investigations proposed that the *E. coli*-expressed RBD of SARS-CoV S protein could create a protective immunity (19,20). Furthermore, as a cost-effective antigen, the *E. coli*-expressed $RBD_{N318-V510}$ of SARS-CoV-2 has been employed for serological COVID-19 testing (21). Bacterial expression systems for heterologous protein expression have the advantages of low cost, easy use, fast-growing, scalability, and short generation times. SARS CoV-2 RBD in *E. coli* lacks glycosylation and disulfide bonding, which makes it more soluble and stable (15).

In this study, the *E. coli* BL21 (DE3) strain was employed to express the RBD domain of SARS-CoV-2, and in the further step, the purification was performed *via* nickel-nitriloacetic acid (Ni-NTA) column. The binding capacity of recombinant RBD to ACE2 was measured at molecular and cellular levels. The outcomes suggest that the *E. coli*-expressed recombinant RBD can be used for developing COVID-19 test kits, screening drugs, and vaccine design.

MATERIALS AND METHODS

Materials

TOP10 and BL21 (DE3) *E. coli* strains were utilized as hosts for cloning and expression of recombinant RBD, respectively. ACE2 was purchased from Sino Biological (Cat. No. 10108-H08H, Beijing, China). The pET-22b (+) vector (Novagen, USA) was used to clone the RBD protein gene downstream of the T7 promoter. Isopropyl ß-D-1-thiogalactopyranoside (IPTG) and pre-stained protein ladder were purchased from Sinaclon, Iran. Polymerase chain reaction (PCR) Master Mix was obtained from Viragen, Iran. DNA ligase, unstained protein marker, DNA ladder, *Xho*I, and *Nde*I were purchased from Fermentas, Germany. All chemicals and reagents were of analytical grade and obtained from Chem-Lab NV, Belgium. The human embryonic kidney cell line (HEK293A) was purchased from the National Cell Bank of Iran. The ethics code of this research is IR.PII.REC.1399.014.

Construct design and cloning

The RBD coding sequence of SARS-CoV-2 S protein (amino acid 319-541, NCBI accession: NC_045512) was amplified by PCR (pFu enzyme) using specific primers (RBDopti

forward: AGC CAT ATG CGC GTT CAG CCG ACC G and RBDopti reverse: AGC CTC GAG CAC GCC GGT GCC GG) from S gene of SARS-Cov-2 virus (Wuhan subspecies; NC_045512) which was synthesized and cloned in a plasmid. PCR was accomplished *via* a thermal cycler (Eppendorf 5333 Mastercycler) as follows: (1) initial DNA denaturation at 95 °C for 5 min, (2) 35 cycles of 95 °C/30 s, 60 °C/45 s, 72 °C/30 s, (3) and final extension at 72 °C for 10 min. The RBD sequence was cloned in pET-22b (+) expression vector using *Nde*I and *Xho*I restriction sites with a C-terminal His-tag, in which, the expression was induced by IPTG and regulated under T7 promoter. After cloning, the construct was transformed into *E. coli* (BL21) and screening the colony that has the gene by colony PCR. Restriction digestion and sequencing were employed to confirm the correct sequence. The resulting plasmid was transformed into BL21 (DE3) *E. coli* strain using the calcium chloride heat-shock method.

Expression of recombinant RBD in E. coli

The protein expression was optimized by different IPTG concentrations (0.1, 0.3, 0.5, and 0.7 mM) and different harvesting times after induction (6 and 16 h). The clone was cultured in 150 mL LB medium overnight and then transferred to 500 mL Erlenmeyer flasks containing LB medium plus 50 µg/mL ampicillin. The culture was incubated at 37 ℃ with an agitation of 250 rpm till the density at 600 nm reached 0.6. Then, 0.7 mM IPTG in the final concentration was added (840 µL from 100 mM stock) to the culture and incubated for 6 h at 37 ℃ with an agitation of 250 rpm. After centrifugation at 4000 *g* for 15 min/4 ℃, the cells were harvested and stored at -20 ℃ for further processing. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (15%, SDS-PAGE) was used to evaluate and optimize the expression condition.

Purification of recombinant RBD

His-tag-mediated affinity chromatography has been used for purification with the Ni-NTA column (Qiagen, Germany). In this method, the pellets from IPTG-induced cells were re-suspended in phosphate-buffered saline (PBS, pH 7.4) containing 100 mM NaCl in a proportion of 7.5 mL per gram of cells (wet weight) and lysed for 30 min using ultrasonication (7 s pulse and 3 s rest). The cell lysates were centrifuged at 10000 *g* at 4 ℃ for 30 min. The pellet containing the inclusion bodies was washed with lysis buffer (0.1% Triton® X-100). After washing with sterile H_2O twice, the precipitate was dissolved in column-binding buffer (8 M urea, 100 mM tris, and 10 mM Na_2HPO_4) and the recombinant protein was finally purified using a Ni-NTA column. SDS-PAGE (15%) was used for analysis of recombinant protein. Finally, the expression was confirmed by western blotting with different antibodies and the protein concentration was obtained by the Bradford method.

Refolding

Fractions containing purified recombinant RBD were combined, and the refolding was conducted in dialysis buffer ((0.5 M NaCl, 1 mM 2-mercaptoethanol, 20 mM tris-HCl, pH 8.0 comprising a serial dilution of urea (0, 2, 4, and 8 M)). To simplify the refolding procedure, renaturation was performed under a gradually decreased concentration of urea (every 2 h). Then, the refolded protein was dialyzed by a membrane (cutoff point of 12 KDa) overnight against PBS (pH 7.4). Finally, the dialyzed proteins were centrifuged at 10000 *g* at 4 ℃ for 10 min to remove the aggregated material and the purity was evaluated using 12% SDS-PAGE. The protein concentration was determined by the Bradford method.

Binding capacity assessments by ELISA

Standard commercial 96-well micro-assay plates were used for ELISA. The recombinant RBD (0.1 µg) in PBS was dispensed into each well and incubated overnight at 4 °C. Next, the wells were treated with 4% skim milk, incubated overnight at 4 °C, and then washed three times with PBS. Human anti-RBD antibodies obtained from the serum of convalescent patients diagnosed as COVID-19 positive (gifted from the Department of Virology, Pasteur Institute of Iran), rabbit IgG anti-SARS-CoV-2 RBD, and RBD immunized camel sera (gifted from Laboratory of

Regenerative Medicine and Biomedical Innovations, Pasteur Institute of Iran) in serial dilution of (1/2, 1/4, 1/8, 1/16, and 1/32) were added to the wells and the plates were incubated for 1 h at room temperature followed by three washes with PBS. In a further step, the plates were incubated with goat anti-rabbit IgG antibody, horseradish peroxidase (HRP, 1:2000), rabbit anti-human IgG (HRP, 1:2000), or rabbit anti-camel IgG antibody (HRP, 1:5000) at room temperature for 1 h followed by three washes with PBS. The positive wells were screened by adding 3,3′,5,5′-tetramethylbenzidine (TMB) solution (100 µL). The enzymatic reaction was stopped by 2 N sulfuric acid, and the absorbance was measured at a wavelength of 450 nm in a BioTek microplate reader (Winooski, VT, US).

Western blotting

For western blotting commercial ACE2 protein was electrophoresed on 15% SDS-PAGE gel and transferred on nitrocellulose paper. The blocking was performed with 4% skim milk at 4 °C overnight. After washing the paper with PBS, the membrane was exposed to 2 µg recombinant RBD protein and incubated overnight at room temperature. After washing with PBS, the membrane was incubated with HRP conjugated 6× His-tag rabbit antibody for 5 h at room temperature and the band visualization was done by adding 3,3'-diaminobenzidine (DAB) as substrate.

Binding evaluation to ACE2 via flow cytometry

The binding potential of recombinant RBD to the ACE2 receptor expressed on the cell surface of the human embryonic kidney cell line (HEK293A) was assessed *via* flow cytometry. The cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37 °C. The cells were then washed 2 times with cold PBS-2% FBS and aliquoted up to 5×10^4 cells per mL. Next, the cells were incubated with 2 µg purified recombinant RBD for 40 min at 4 ℃ and then with 1 µg rabbit anti-6-his tag antibody for 40 min at 4 °C. To screen the conjugated molecules, 1 µg goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated was added for 40 min at 4 °C. The commercial antibody against ACE2 (Abcam, ab15348, UK) was used as the positive control. Finally, the cells were washed, and the fluorescent intensity was measured using the FL1 channel of a flow cytometer (Cyflow, Partec, Germany). Data interpretation was performed by FlowJo software v. 7.6 (Tree Star, USA).

Statistical analysis

To assess the significance of the binding, the absorbance values from the experimental groups were compared to those of the control group using a one-way ANOVA followed by tukey's multiple comparisons test. The *P*-values of less than 0.05 were considered statistically significant, indicating that the recombinant RBD had a strong binding affinity for both human anti-RBD antibodies and rabbit anti-SARS-CoV-2 RBD antibodies. The statistical tests were performed using GraphPad Prism 10, USA.

RESULTS

Construct design and cloning

After cloning the sequence of RBD in the pET-22b (+) expression vector, the colony PCR was performed using T7 promoter and T7 terminator primers and a band with a size of 700 bp was obtained (Fig. 1). The plasmid was then extracted and confirmed by sequencing and enzymatic digestion with *Nde*I and *Xho*I restriction enzymes, where a linear plasmid of ~ 5000 bp and a band of 700 bp was detected in the agarose gel (Fig. 2).

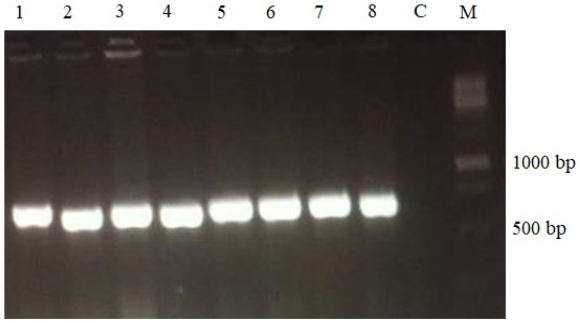

Fig. 1. Eight colonies were selected for colony polymerase chain reaction which yielded a band of 700 bp. C, Negative control; M, DNA ladder.

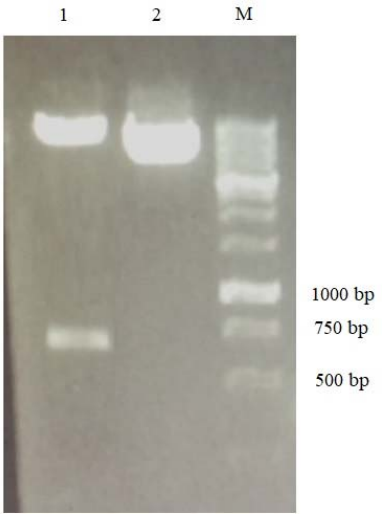

Fig. 2. Confirmation by enzymatic digestion of recombinant plasmid. Lane 1 represents the digested recombinant plasmid; lane 2, the undigested recombinant plasmid; and lane M, the DNA ladder.

RBD expression

To optimize the expression yield, different concentrations of IPTG, induction temperature, and time of bacterial harvest were examined. Based on the outcomes, *E. coli* BL-21 (DE3) displayed the highest expression at 0.7 mM IPTG and 6-h incubation (Fig. 3). The inclusion bodies containing RBD were consequently dissolved, renatured, and purified by the Ni-NTA column. After purification, the SDS-PAGE analysis (Fig. 4) revealed the corresponding band at 27 kDa in the culture after induction with 0.7 mM IPTG, while the corresponding band was not observed in the culture without IPTG induction. After purification the protein concentration was measured by Bradford assay and the yield was 6 mg/L of the culture medium. Western blot method with antibody against His-tag was used to determine the characteristics of recombinant RBD. As shown in Fig. 5A, the 27 kDa band appeared on the paper. Western blotting was also used to check the RBD's ability to identify ACE2. In this method, recombinant ACE2 was loaded on nitrocellulose paper and then RBD and secondary antibodies were added. As shown in Fig. 5B, western blotting has the potential for recognizing ACE2 by RBD.

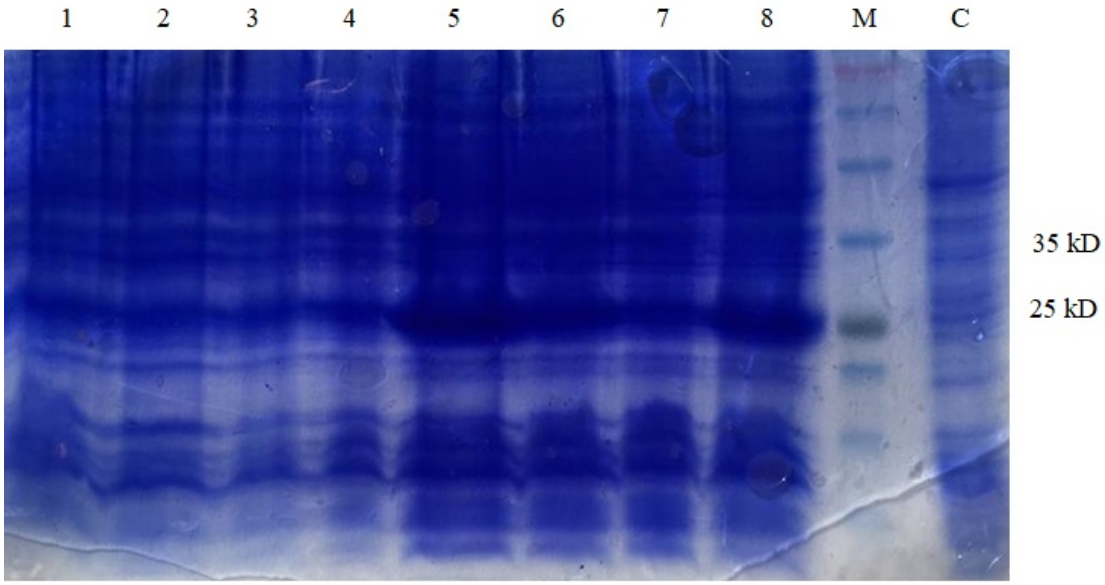

Fig. 3. Expression optimization of receptor binding domain recombinant protein illustrated on SDS-PAGE. Lane 1, 0.7 mM IPTG and 16-h incubation; lane 2, 0.3 mM IPTG and 16-h incubation; lane 3, 0.5 mM IPTG and 16-h incubation; lane 4, 0.1 mM IPTG and 16-h incubation; lane 5, 0.7 mM IPTG and 6-h incubation; lane 6, 0.3 mM IPTG and 6-h incubation; lane 7, 0.5 mM IPTG and 6-h incubation; lane 8, 0.1 mM IPTG and 6-h incubation; M, protein marker; C, negative control (before induction with IPTG). IPTG, Isopropyl ß-D-1-thiogalactopyranoside.

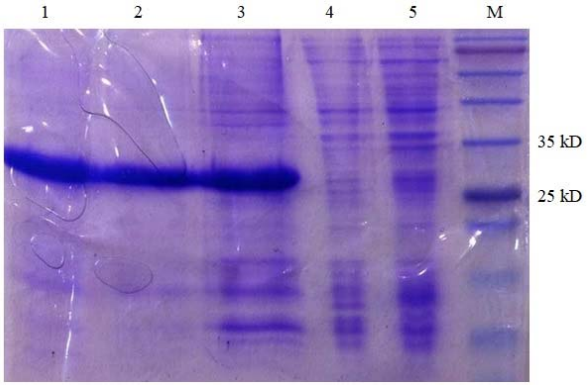

Fig. 4. Characterization of expressed recombinant RBD by SDS-PAGE. Lanes 1 and 2, purified recombinant RBD; lane 3, induced bacterial cell lysate; lane 4, non-induced bacterial cells lysate; lane 5, washing elute from Ni-NTA column; M, protein marker; RBD, receptor binding domain.

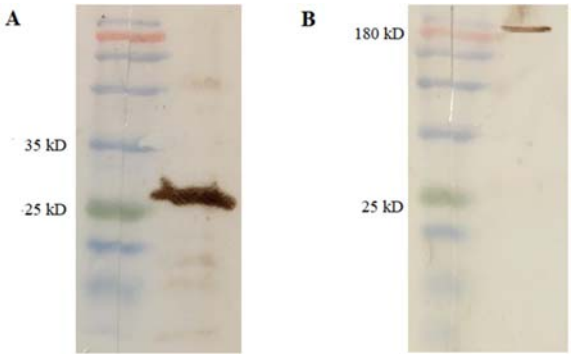

Fig. 5. Characterization of expressed recombinant RBD by western blotting. (A) Western blot of RBD protein using anti-His-tag rabbit antibody; (B): detection of commercial human ACE2 by recombinant RBD. RBD, receptor binding domain.

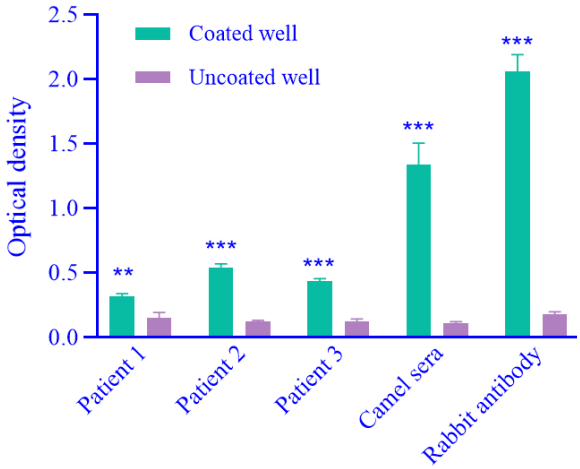

Fig. 6. The reaction between the recombinant RBD and rabbit IgG anti-SARS-CoV-2 RBD resulted in a significant positive response at 450 nm. An immune response to the RBD was detected in the sample of immunized camel serum. Antibodies from the serum of convalescent patients diagnosed as COVID-19 positive showed binding ability to recombinant RBD. The $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ indicate significant differences compared to the respective control group (uncoated wells). RBD, receptor binding domain.

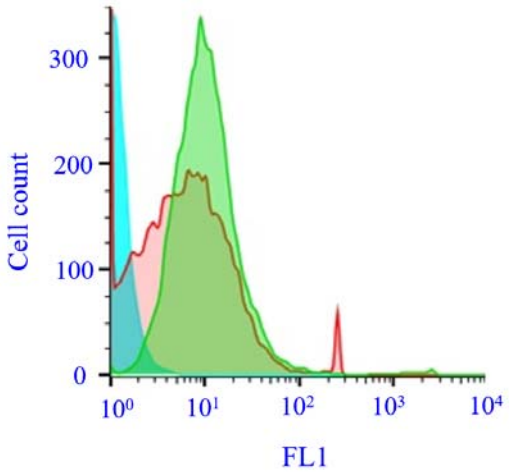

Fig. 7. Detection of RBD binding activity on human ACE2 receptor expressed in HEK293A cells by flow cytometry. The green signal corresponds to cells expressing ACE2 interacting with RBD. The red signal corresponds to commercial anti-ACE2 interacting with the ACE2 receptor expressed in HEK293A and the cyan signal corresponds to unlabeled ACE2/HEK293A cells. RBD, receptor binding domain; ACE2, angiotensin-converting enzyme 2.

Binding capacity evaluation using ELISA and flow cytometry

The binding capability of recombinant RBD protein was studied by different antibodies including human anti-RBD antibodies obtained from the serum of convalescent patients (diagnosed as COVID-19 positive), rabbit IgG anti-SARS-CoV-2 RBD, and HRP conjugated 6× His-tag rabbit antibody. The ELISA elucidated a significant positive reaction between the recombinant RBD and rabbit anti-SARS-CoV-2 RBD at 450 nm. An immune response to the RBD in the samples of immunized camel sera was also detected. Anti-SARS-CoV-2 antibodies from the serum of convalescent patients showed the binding ability for the recombinant RBD based on the concentration of serum antibodies (Fig. 6).

To evaluate the specific binding of recombinant RBD on ACE2 expressing HEK293A cells, a flow cytometry assay was conducted. The outcomes demonstrated that recombinant RBD exhibited a selective interaction with ACE2 receptors on the surface of HEK293A cells. This interaction closely mirrored the performance of the commercial antibody used as a positive control, thereby validating the efficacy of the recombinant RBD in recognizing and binding to the ACE2 receptors (Fig. 7).

DISCUSSION

Since the COVID-19 pandemic, the mechanisms of SARS-CoV-2 entry into the host cells have been extensively studied. ACE2 receptors are specifically detected by the RBD domain in the S1 subunit of SARS-CoV. Similarly, RBD in SARS-CoV-2 detects human ACE2 with a much higher affinity (22). Several SARS-CoV-2 vaccine candidates that use RBD as the antigen have been designed lately, and recombinant RBD is an effective and safe candidate for developing vaccines and diagnostic kits (8). Currently, several expression systems are employed for the production of recombinant SARS-CoV-2 RBD, focusing mainly on eukaryotic expression systems (23). A major advantage of eukaryotic expression systems is that they fold properly and undergo post-translational modifications. However, proteins derived from the eukaryotic cells suffered from low expression yield and high cost (24). The present cost of 1 µg commercially available S1-derived SARS-CoV-2 antigens is about 7 USD, which is high for the majority of laboratories conducting large-scale screening of COVID-19 (21).

Recently the RBD protein was produced in *E. coli* Rosetta (DE) *via* Gao *et al.* and purified by a Ni-NTA column. SDS-PAGE and western blot investigation of the recombinant RBD was carried out. The flow cytometry evaluation showed that the recombinant RBD is able to bind to human ACE2 (hACE2) in the ACE2-overexpressed HEK293A-hACE2 cells. Their outcomes displayed that recombinant RBD expressed in the *E. coli* Rosetta (DE) strain is bioactive and can be employed as a tool for the design of novel anti-viral drugs against SASR-CoV-2 and as an antigen for diagnosis (25).

García-Cordero and collogues developed the expression of the recombinant S1 and N proteins from SARS-CoV-2 within a mammalian system utilizing the plasmids pcDNA3.1/S1 and pcDNA3.1/N, which has been later improved. The RBD was used as a control. Expi293 cells were effectively used to isolate the antigens, and large yields of the S1, N, and RBD proteins were obtained. These proteins' immunogenic properties were proven in a mouse model. The SARS-CoV-2 antigens are a good replacement for serological assays to identify individuals infected with COVID-19, as demonstrated by the ELISA technique using human serum samples (26).

Here, we report a simple method for the production of recombinant RBD *via* expression in *E. coli* in the form of inclusion bodies. The expression of RBD was optimized and harvested after 6 h in inclusion bodies form with high yield, easily dissolved in 0.1% SDS, and renatured in the presence of urea. According to the findings of the current study a functional RBD can be achieved with a high yield and purity. *E. coli* was employed as an expression host due to a simple process and widely applicable for the preparation of recombinant RBD. The pET-22b (+) vector was utilized which is ampicillin resistant and can reduce the costs. Also, the codon optimization at our cloning step replaced the rare codons with preferred codons to enhance the expression level of protein in *E. coli*. Expression conditions have also been optimized to increase the expression yield. The bacterial system employed in the current significantly streamlines production procedures thereby facilitating industrial-scale manufacturing. The antigen yield of 6 mg/L of culture medium was achieved without utilizing instrumented bioreactors. The production can be efficiently completed in 24 h. Nevertheless, the use of a bacterial system for the expression of recombinant proteins always has some drawbacks. Bacterial systems cannot add post-translational modifications such as glycosylation, disulfide bond formation, and phosphorylation which is mainly essential for proper function. Consequently, recombinant

proteins expressed in bacteria may have a lower functional capacity than analogous proteins derived from eukaryotes. (27). In the present study ELISA assay revealed that the *E. coli* expressed recombinant RBD preserves its ability to bind to anti-RBD antibodies. The obtained results by flow cytometry indicated that the *E. coli*-expressed recombinant RBD was able to detect the ACE2 receptor on the surface of ACE2-expressing cells. Furthermore, the assessment of the HEK293A cells' surface by flow cytometry revealed that the ACE2 binding ability of *E. coli*-expressed recombinant RBD is comparable with the commercial rabbit polyclonal ACE2 antibody.

CONCLUSION

This study presents an efficient process for preparing functional recombinant RBDs using the pET expression system and *E. coli* strain BL21 (DE3). Recombinant RBD produced in this study showed an effective binding ability in ELISA assay. More importantly, the expressed recombinant RBD can detect and bind to human ACE2 receptors on the surface of HEK293A cells. It can be used to develop diagnostic kits, screen drugs for potential efficacy, and develop vaccines based on the recombinant RBD produced in this study.

Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

H. Ghaderi and M. Behdani designed the research; H. Ghaderi, S. Salehi, and A. Hassanzadeh Eskafi performed the research; M. Habibi-Anbouhi and R. Ahangari Cohan analyzed the data; R. Moazzami and R. Ahangari Cohan reviewed and revised the paper; M. Behdani and A. Shoari drafted, reviewed, and revised the paper. The finalized article was read and approved by all authors.

REFERENCES

1. Fatemi B, Rezaei S, Peikanpour M, Dastan F, Saffaei A. Efficacy of intravenous immunoglobulins (IVIG) in COVID-19 patients: a systematic review and meta-analysis. Res Pharm Sci. 2023;18(4): 346-357. DOI: 10.4103/1735-5362.378082.
2. Modig K, Ahlbom A, Ebeling M. Excess mortality from COVID-19: weekly excess death rates by age and sex for Sweden and its most affected region. Eur J Public Health. 2021;31(1):17-22. DOI: 10.1093/eurpub/ckaa218.
3. Wang B, Zhang L, Wang Y, Dai T, Qin Z, Zhou F, *et al*. Alterations in microbiota of patients with COVID-19: potential mechanisms and therapeutic interventions. Signal Transduct Target Ther. 2022;7(1):1-15. DOI: 10.1038/s41392-022-00986-0.
4. Sarkesh A, Daei Sorkhabi A, Sheykhsaran E, Alinezhad F, Mohammadzadeh N, Hemmat N, *et al*. Extrapulmonary clinical manifestations in COVID-19 patients. Am J Trop Med Hyg. 2020;103(5):1783-1796. DOI: 10.4269/ajtmh.20-0986.
5. Satarker S, Nampoothiri M. Structural proteins in severe acute respiratory syndrome coronavirus-2. Arch Med Res. 2020;51(6):482-491. DOI: 10.1016/j.arcmed.2020.05.012.
6. Huang Y, Yang C, Xu XF, Xu W, Liu SW. Structural and functional properties of SARS-CoV-2 spike protein: potential antivirus drug development for COVID-19. Acta Pharmacol Sin. 2020;41(9):1141-1149. DOI: 10.1038/s41401-020-0485-4.
7. Jackson CB, Farzan M, Chen B, Choe H. Mechanisms of SARS-CoV-2 entry into cells. Nat Rev Mol cell Biol. 2022;23(1):3-20. DOI: 10.1038/s41580-021-00418-x.
8. Min L, Sun Q. Antibodies and vaccines target RBD of SARS-CoV-2. Front Mol Biosci. 2021;8:671633,1-9. DOI: 10.3389/fmolb.2021.671633.
9. Shin YJ, König-Beihammer J, Vavra U, Schwestka J, Kienzl NF, Klausberger M, *et al*. N-glycosylation of the SARS-CoV-2 receptor binding domain is important for functional expression in plants. Front Plant Sci. 2021;12:1154,1-14. DOI: 10.3389/fpls.2021.689104.
10. Farnós O, Venereo-Sánchez A, Xu X, Chan C, Dash S, Chaabane H, *et al*. Rapid high-yield production of functional SARA-coV-2 receptor binding domain by viral and non-viral transient expression for pre-clinical evaluation. Vaccines. 2020;8(4): 654,1-20. DOI:10.3390/vaccines8040654.
11. Gong Y, Qin S, Dai L, Tian Z. The glycosylation in SARS-CoV-2 and its receptor ACE2. Signal Transduct Target Ther. 2021;6(1):396,1-24. DOI: 10.1038/s41392-021-00809-8.
12. Watanabe Y, Allen JD, Wrapp D, McLellan JS, Crispin M. Site-specific glycan analysis of the SARS-CoV-2 spike. Science. 2020;369(6501):330-331. DOI: 10.1126/science.abb9983.
13. Chen WH, Pollet J, Strych U, Lee J, Liu Z, Kundu RT, *et al*. Yeast-expressed recombinant SARS-CoV-2 receptor binding domain RBD203-N1 as a COVID-19 protein vaccine candidate. Protein Expr Purif. 2022;190:106003,1-10. DOI: 10.1016/j.pep.2021.106003.

14. De March M, Terdoslavich M, Polez S, Guarnaccia C, Poggianella M, Marcello A, *et al*. Expression, purification and characterization of SARS-CoV-2 spike RBD in ExpiCHO cells. Protein Expr Purif. 2022;194:106071,1-6. DOI: 10.1016/j.pep.2022.106071.
15. He Y, Qi J, Xiao L, Shen L, Yu W, Hu T. Purification and characterization of the receptor-binding domain of SARS-CoV-2 spike protein from *Escherichia coli*. Eng Life Sci. 2021;21(6):453-460. DOI: 10.1002/elsc.202000106.
16. Fan Y, Wei J, Wang W, Li C, Pan G, Keiffer T, *et al*. Utilization of recombinant Baculovirus expression system to produce the RBD domain of SARS-CoV-2 spike protein. Pathogens. 2022;11(6):672,1-9. DOI: 10.3390/pathogens11060672.
17. Khow O, Suntrarachun S. Strategies for production of active eukaryotic proteins in bacterial expression system. Asian Pac J Trop Biomed. 2012;2(2): 159-162. DOI: 10.1016/S2221-1691(11)60213-X.
18. Sadeghian-Rizi T, Ebrahimi A, Moazzen F, Yousefian H, Jahanian-Najafabadi A. Improvement of solubility and yield of recombinant protein expression in *E. coli* using a two-step system. Res Pharm Sci. 2019; 14(5): 400-407. DOI: 10.4103/1735-5362.268200.
19. Chen J, Miao L, Li JM, Li YY, Zhu QY, Zhou CL, *et al*. Receptor-binding domain of SARS-Cov spike protein: soluble expression in *E. coli*, purification and functional characterization. World J Gastroenterol. 2005;11(39):6159-6164. DOI: 10.3748/wjg.v11.i39.6159.
20. Du L, Zhao G, Chan CCS, Sun S, Chen M, Liu Z, *et al*. Recombinant receptor-binding domain of SARS-CoV spike protein expressed in mammalian, insect and *E. coli* cells elicits potent neutralizing antibody and protective immunity. Virology. 2009;393(1): 144-150. DOI: 10.1016/j.virol.2009.07.018.
21. Márquez-Ipiña AR, González-González E, Rodríguez-Sánchez IP, Lara-Mayorga IM, Mejía-Manzano LA, Sánchez-Salazar MG, *et al*. Serological test to determine exposure to SARS-CoV-2: ELISA based on the receptor-binding domain of the spike protein (S-RBDN318-V510) expressed in *Escherichia coli*. Diagnostics. 2021;11(2):271,1-15. DOI: 10.3390/diagnostics11020271.
22. Yazdani M, Khezri J, Hadizadeh N, Zamani Amir Zakaria J, Naderi M, Mahmoodian S, *et al*. Depinar, a drug that potentially inhibits the binding and entry of COVID-19 into host cells based on computer-aided studies. Res Pharm Sci. 2021; 16(3):315-325. DOI: 10.4103/1735-5362.314830.
23. Cabal ABS, Wu TY. Recombinant protein technology in the challenging Era of coronaviruses. Processes. 2022;10(5):946,1-24. DOI: 10.3390/pr10050946.
24. Azadi S, Sadjady SK, Mortazavi SA, Naghdi N, Mahboubi A, Solaimanian R. Bioprocess and downstream optimization of recombinant human growth hormone in *Pichia pastoris*. Res Pharm Sci. 2018;13(3):222-238. DOI: 10.4103/1735-5362.228953.
25. Gao X, Peng S, Mei S, Liang K, Khan MSI, Vong EG, *et al*. Expression and functional identification of recombinant SARS-CoV-2 receptor binding domain (RBD) from *E. coli* system. Prep Biochem Biotechnol. 2022;52(3):318-324. DOI: 10.1080/10826068.2021.1941106.
26. Garcia-Cordero J, Mendoza-Ramirez J, Fernandez-Benavides D, Roa-Velazquez D, Filisola-Villasenor J, Martinez-Frias SP, *et al*. Recombinant protein expression and purification of N, S1, and RBD of SARS-CoV-2 from mammalian cells and their potential applications. Diagnostics. 2021;11(10):1808,1-14. DOI: 10.3390/diagnostics11101808.
27. Chen R. Bacterial expression systems for recombinant protein production: *E. coli* and beyond. Biotechnol Adv. 2012;30(5):1102-1107. DOI: 10.1016/j.biotechadv.2011.09.013.